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In vitro effect of pentoxifylline and lisofylline on deformability and aggregation of red blood cells from healthy subjects and patients with chronic venous disease

Karolina Słoczyńska[1]✉, Mariusz Kózka[2], Elżbieta Pękala[3], Anna Marchewka[4] and Henryk Marona[1]

[1]Department of Bioorganic Chemistry, Chair of Organic Chemistry, Faculty of Pharmacy, Jagiellonian University Medical College, Cracow, Poland; [2]5th Military Hospital with Polyclinic, Cracow, Poland; [3]Department of Pharmaceutical Biochemistry, Faculty of Pharmacy, Jagiellonian University Medical College, Cracow, Poland; [4]Department of Clinical Rehabilitation, University School of Physical Education, Cracow, Poland

Purpose. **The aim of the study was to assess the *in vitro* potency of pentoxifylline (PTX) and one of its most active metabolites lisofylline (LSF) to improve rheological properties of red blood cells (RBC) from healthy individuals and patients with chronic venous disease (CVD). Additionally, the study aimed to compare the effects of PTX and LSF on RBC deformability and aggregation. Methods**. **Blood samples were collected from healthy volunteers (antecubital vein) and from CVD patients (varicose and antecubital vein). Deformability and aggregation of RBC were assessed using Laser-assisted Optical Rotational Cell Analyser (LORCA). Results**. **PTX and LSF increased RBC elongation significantly. Additionally, RBC incubation with PTX resulted in a marked decrease in RBC aggregation. PTX reduced the tendency towards the formation of RBC aggregates and of their stability. The beneficial effect of PTX on RBC aggregation was most apparent for those cells whose aggregation tendency and aggregate stability was the greatest. Conclusions**. ***In vitro*** **addition of PTX or LSF effectively increased deformability of RBC from healthy donors and patients with CVD. Thus, LSF may contribute to the *in vivo* hemorheological effects of pentoxifylline. On the other hand, there was no significant effect of LSF on aggregation of RBC *in vitro*. Hence, LSF has no contribution to this particular effect of PTX. Additionally, the present study demonstrated the use of RBC with impaired deformability and aggregation for the evaluation of *in vitro* rheological activity of xenobiotics.**

Key words: pentoxifylline, lisofylline, red blood cells, deformability, aggregation

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INTRODUCTION

Pentoxifylline (PTX) is a xanthine derivative and a nonspecific inhibitor of cyclic adenosine monophosphate (AMP) phosphodiesterases, widely used in daily clinical practice for treatment of various cerebrovascular and peripheral vascular diseases characterized by a defective tissue perfusion (Moher *et al*., 2000; Jull *et al*., 2002). The therapeutic effect of PTX is related mainly to its ability to improve microvascular blood flow. The compound has been reported to increase flexibility of red blood cells (RBC) and to decrease blood viscosity (Muller, 1981; Ott *et al*. 1983; Eun *et al*., 2000). Additionally, it was demonstrated that PTX reduces RBC aggregation (Accetto, 1982). As a hemorheological agent the compound acts also by decreasing the potential for platelet aggregation and thrombus formation (Frampton & Brodgen, 1995).

Several studies have been conducted to investigate the action of PTX *in vitro* and *in vivo* on hemorheology. However, no *in vivo* effect of PTX or its metabolites on RBC rheology has been found. Thus, the aim of the present study was to assess the *in vitro* potency of pentoxifylline and one of its most active metabolites, lisofylline (LSF), to improve rheological properties of erythrocytes from healthy individuals (antecubital vein) and patients with chronic venous disease (CVD) (antecubital vein and varicose vein), and to compare the effects of PTX and LSF on RBC deformability and aggregation.

MATERIALS AND METHODS

Blood samples. Blood samples were collected from healthy volunteers ($n = 30$; mean age 39.46 ± 15.23 years) and from CVD patients with varicosis ($n = 26$; mean age 44.64 ± 14.75 years) *via* withdrawal into potassium EDTA (ethylenediaminetetraacetic acid) containing tubes. The patients were rated as having lesions of CVD levels II and III according to clinical features included in the CEAP (clinical, etiological, anatomical and pathological elements) classification. The patients considered for the study were those attending the 2nd Chair of General Surgery of the Jagiellonian University Medical College for the management of venous disease. The diagnosis of primary varicose vein was based on the clinical examination and duplex scanning examination. The patients had a positive family history of CVD and the symptoms of the disease had been observed for more than one year. Additionally, all patients showed normal fibrinogen level. Patients had not taken any medication within the last two weeks prior to blood withdrawal. Blood was collected from antecubital veins of healthy subjects and

✉e-mail: karolina.sloczynska@uj.edu.pl

Abbreviations: AI, aggregation index; AMP, adenosine monophosphate; ATP, adenosine triphosphate; CEAP, clinical, etiological, anatomical and pathological elements; CVD, chronic venous disease; EDTA, ethylenediaminetetraacetic acid; EI, elongation index; LORCA, Laser-assisted Optical Rotational Cell Analyser; LSF, lisofylline; M, metabolite; PBS, phosphate buffered saline; PTX, pentoxifylline; PVP, polyvinylpyrrolidone; RBC, red blood cells; T1/2, aggregation halftime; THR, threshold shear rate.

from antecubital veins and varicose veins of CVD patients. All volunteers gave their informed consent prior to donating their blood. The study was approved by the Commission of Bioethics of the Jagiellonian University.

Reagents. PTX was purchased from Sigma. LSF was obtained through enantioselective reduction of PTX using whole-cell *Lactobacillus kefiri* according to Pękala *et al.* (2007). All drugs were diluted with phosphate buffered saline (PBS).

Incubation with drugs. The blood samples were centrifuged (1400 × *g*, 5 min, 4°C), blood plasma was removed and the remaining erythrocytes were washed 3 times with PBS (pH = 7.4). The washed RBC were then re-suspended in autologous plasma at a hematocrit of 40% (Baskurt *et al.*, 2009). RBC suspensions were divided into aliquots and exposed to one of the drugs (at a final concentration of 10^{-4} and 10^{-5} M) or PBS (control sample) at 37°C for 30 minutes. The samples were incubated with constant mixing in a water bath. After incubation in the drug solution, RBC were washed as above and re-suspended in autologous plasma at a hematocrit of 40%. The morphology of RBC was evaluated using a Leica DM1000 microscope (Leica Microsystems) and RBC deformability and aggregation were assessed.

Deformability measurement. RBC deformability was measured with a Laser-assisted Optical Rotational Cell Analyser (LORCA, Mechatronics, The Netherlands), according to the methods of Hardeman *et al.* (1994 and 2001). Twenty-five μl of blood was diluted 200 times with 0.14 mM polyvinylpyrrolidone (PVP, pH = 7.4, osmotic pressure 300 mOsm/kg, viscosity 30 mPas, Sigma). RBC deformability was analyzed at a range of shear stresses (0.30–59.97 Pa) and expressed as elongation index (EI) of erythrocytes defined as EI = (A–B)/(A+B), where A and B are the long and short axes of the ellipse, respectively (Hardeman *et al.*, 1994). The higher the value of EI, the greater deformation of blood cells.

Aggregation measurement. The aggregation of erythrocytes was also approached by LORCA. Aggregation measurement was performed using 1 to 2 ml blood. Erythrocytes were oxygenated for 10–15 minutes before the measurement by slow rotation in a glass vessel. The aggregation measurement was based on the detection of laser back-scattering from sheared, then unsheared blood. RBC aggregation parameters were determined with a syllectogram, which is a curve illustrating the change in the light intensity of scattered light during 120 s corresponding to the process of aggregation (Hardeman *et al.*, 2001). The following parameters of aggregation were studied: aggregation index (AI), threshold shear rate (THR), and aggregation halftime (T½). AI characterizes the extent of RBC aggregation, T½ reflects aggregation kinetics whereas THR shows the tendency towards the formation of aggregates and their stability. An increase in THR means an increased tendency towards the formation of aggregates and of their stability. A drop in T½ parameter indicates a faster rate of aggregation (Hardeman *et al.*, 2001).

Statistical analysis and data presentation. Data are presented as means ± S.D. of *n* experiments. Comparisons of the effects of the investigated compounds on the RBC deformability and aggregation were made by statistical analysis of variance (ANOVA). The results were considered significant when $p < 0.05$. Tests were performed using GraphPad Prism version 5.00 for Windows.

RESULTS

RBC deformability

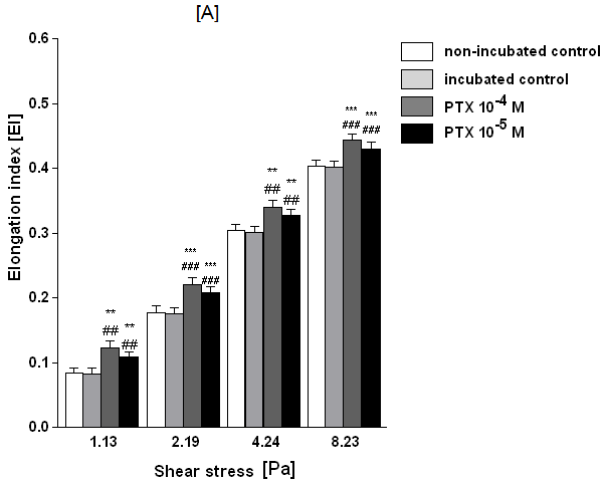
In vitro studies showed that PTX and LSF in both concentrations tested increased the RBC elongation significantly. Graphs depict only those shear stress values for which significant EI changes were observed. Incubation of RBC from healthy adults and CVD patients with PTX led to EI increase *vs* control (Fig. 1A–C). In the case of erythrocytes from healthy donors, a significant improvement of RBC elongation was stated for shear stresses: 1.13 (10^{-4} M 48.8% increase *vs* control, $p < 0.01$; 10^{-5} M 32.9% increase *vs* control, $p < 0.01$), 2.19 (10^{-4} M 26.3% increase *vs* control, $p < 0.001$; 10^{-5} M 18.9% increase *vs* control, $p < 0.001$), 4.24 (10^{-4} M 13% increase *vs* control, $p < 0.01$; 10^{-5} M 9% increase *vs* control, $p < 0.01$) and 8.23 Pa (10^{-4} M 10.7% increase *vs* control, $p < 0.001$; 10^{-5} M 7% increase *vs* control, $p < 0.001$) (Fig. 1A). In the case of RBC from the antecubital vein of CVD patients for shear stress values: 2.19 (17.8% at 10^{-4} M, $p < 0.01$ and 13.9% at 10^{-5} M, $p < 0.01$), 4.24 (13.7% at 10^{-4} M, $p < 0.01$ and 9.5% at 10^{-5} M, $p < 0.01$) and 8.23 Pa (12.5% at 10^{-4} M, $p < 0.001$ and 7.4% at 10^{-5} M, $p < 0.001$) (Fig. 1B). For the RBC from the varicose vein of CVD patients for shear stresses: 2.19 (21.8% at 10^{-4} M, $p < 0.01$ and 12.7% at 10^{-5} M, $p < 0.01$), 4.24 (12.4% at 10^{-4} M, $p < 0.01$ and 8.4% at 10^{-5} M, $p < 0.01$) and 8.23 Pa (10.6% at 10^{-4} M, $p < 0.01$ and 6.9% at 10^{-5} M, $p < 0.01$) (Fig. 1C). When comparing the deformability results between healthy subjects and CVD patients it was found that the EI values obtained in the presence of PTX (in both concentrations tested) were significantly different ($p < 0.05$) for the two groups for shear stress values 1.13 and 8.23 Pa. For shear stresses 2.19 and 4.24 Pa the differences were significant ($p < 0.05$) between RBC from healthy adults and the varicose vein of CVD patients.

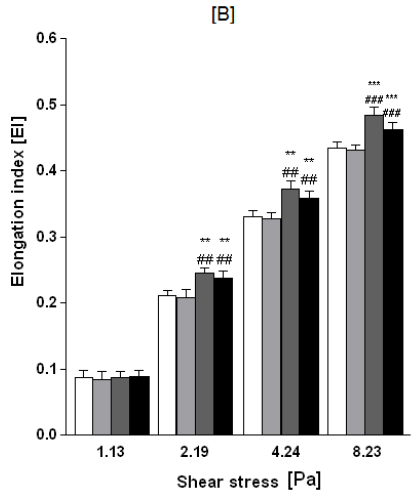
The RBC derived from the three different sources and treated with LSF had improved deformability for shear stress values 4.24 and 8.23 Pa. In the case of healthy donors erythrocytes, a significant improvement of RBC elongation was found for shear stresses: 4.24 (10^{-4} M 17% increase *vs* control, $p < 0.01$; 10^{-5} M 10% increase *vs* control, $p < 0.01$) and 8.23 (10^{-4} M 13.5% increase *vs* control, $p < 0.001$; 10^{-5} M 7% increase *vs* control, $p < 0.001$) (Fig. 2A). For the RBC from the antecubital vein of CVD patients for shear stress values: 4.24 (14% at 10^{-4} M, $p < 0.001$ and 8.8% at 10^{-5} M, $p < 0.001$) and 8.23 Pa (10.7% at 10^{-4} M, $p < 0.01$ and 6.5% at 10^{-5} M, $p < 0.01$) (Fig. 2B). For the RBC from the varicose vein of CVD patients for shear stresses: 4.24 (12.1% at 10^{-4} M, $p < 0.01$ and 8.4% at 10^{-5} M, $p < 0.01$) and 8.23 Pa (8.4% at 10^{-4} M, $p < 0.01$ and 6.2% at 10^{-5} M, $p < 0.01$) (Fig. 2C).

A comparison of the deformability results between healthy donors and patients showed that the EI values obtained in the presence of LSF (in both concentrations tested) differ significantly ($p < 0.05$) for shear stress 2.19 Pa. At shear stress values 4.24 and 8.23 Pa the differences were significant ($p < 0.05$) between the RBC from healthy donors and the varicose vein of CVD patients.

RBC aggregation

Considering the aggregation measurements, PTX improved the RBC aggregation significantly. Incubation of healthy donors and CVD patients' erythrocytes with PTX caused a significant decrease of AI (healthy





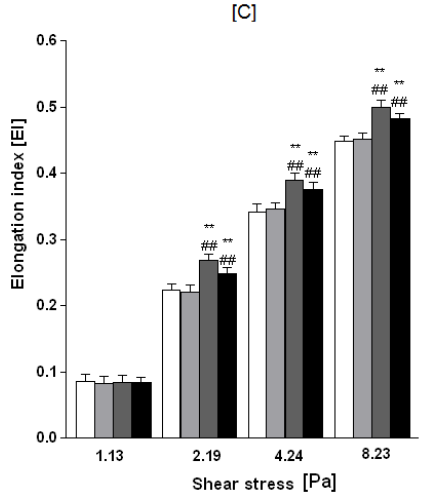
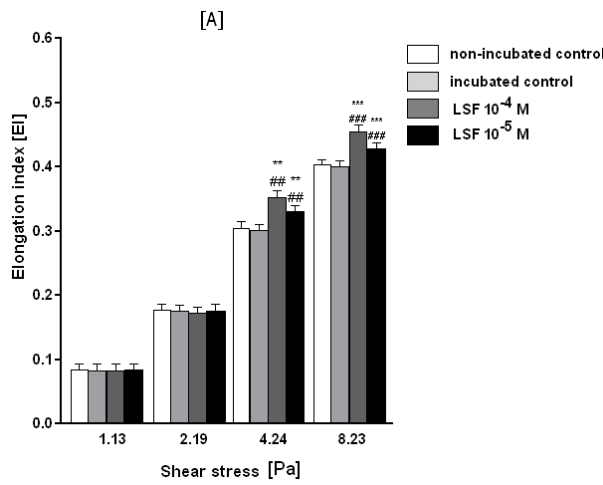
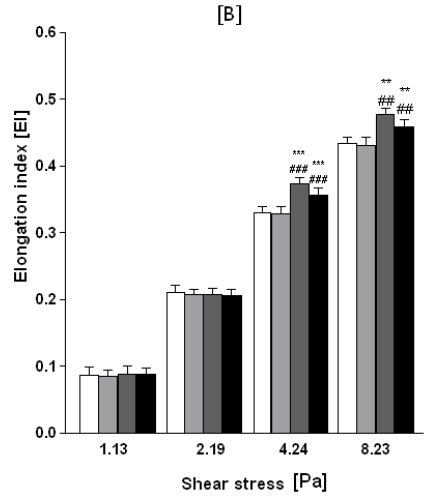


Figure 1. The influence of pentoxifylline (PTX) on RBC deformability.
RBC derived from: (**A**) antecubital vein of healthy donors; (**B**) antecubital vein of CVD patients; (**C**) varicose vein of CVD patients. Data are given as means ± S.D. of n= 10 experiments. Significance *vs* control: $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$; significance *vs* non-incubated control: $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$.





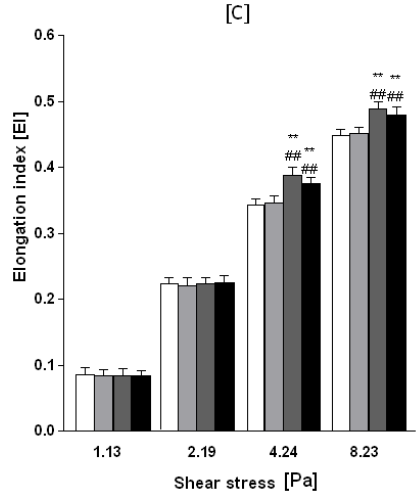


Figure 2. The influence of lisofylline (LSF) on RBC deformability.
RBC derived from: (**A**) antecubital vein of healthy donors; (**B**) antecubital vein of CVD patients; (**C**) varicose vein of CVD patients. Data are given as means ± S.D. of n = 10 experiments. Significance *vs* control: $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$; significance *vs* non-incubated control: $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$.

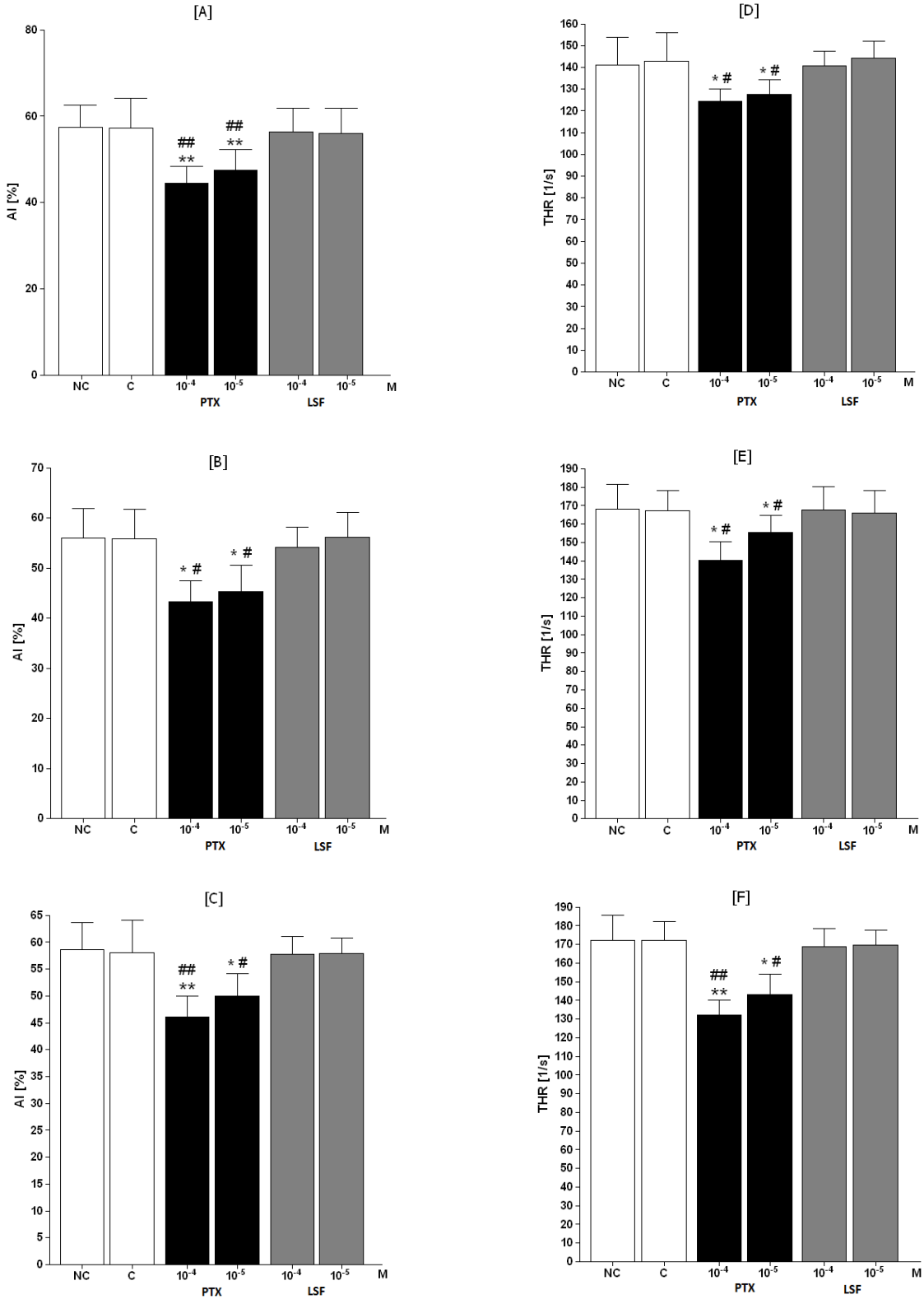


Figure 3.The influence of pentoxifylline (PTX) and lisofylline (LSF) on RBC aggregation index (AI) and threshold shear rate (THR). RBC derived from: (**A**) and (**D**), antecubital vein of healthy donors; (**B**) and (**E**), antecubital vein of CVD patients; (**C**) and (**F**), varicose vein of CVD patients. Data are given as means ±S.D. of *n*= 10 experiments. Significance *vs* control: $^{*}p<0.05$,$^{**}p<0.01$, $^{***}p<0.001$; significance *vs* non-incubated control: $^{\#}p<0.05$,$^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$. NC, non-incubated control; C, control.

donors: –22.5% at 10^{-4} M, $p<0.01$ and –17.3% at 10^{-5} M, $p<0.01$; CVD patients — antecubital vein: –22.7% at 10^{-4} M, $p<0.05$ and –19% at 10^{-5} M, $p<0.05$; CVD patients — varicose vein: –20.7% at 10^{-4} M, $p<0.01$ and –13.9% at 10^{-5} M, $p<0.05$) (Fig. 3A–C). A decrease in THR was observable after treatment of the RBC from healthy donors and CVD patients with either dosage of PTX (healthy donors: –13% at 10^{-4} M, $p<0.05$ and

–10.7% at 10^{-5} M, $p<0.05$; CVD patients — antecubital vein: –16% at 10^{-4} M, $p<0.05$ and –7.1% at 10^{-5} M, $p<0.05$; CVD patients – varicose vein: –23.1% at 10^{-4} M, $p<0.01$ and –16.8% at 10^{-5} M, $p<0.05$) (Fig. 3D–F). A significant improvement of T½ was observed after incubation with either PTX concentration only for RBC from the varicose vein of CVD patients (32.7% at 10^{-4} M, $p<0.05$ and 13.7% at 10^{-5} M, $p<0.05$).

When comparing aggregation parameters between controls and CVD patients it was noted that AI values obtained in the presence of PTX (in lower concentration tested) differ significantly ($p<0.05$) between the RBC from healthy adults and varicose vein of CVD patients. In case of THR significant differences ($p<0.05$) were observed after incubation with PTX (higher concentration) between RBC from controls and antecubital vein of the patients. In case of lower PTX concentration significant differences ($p<0.05$) were noted between RBC from healthy donors and varicose vein samples. The same was found for LSF. Regarding T½ parameter, statistical significance ($p<0.05$) was stated after incubation with higher PTX concentration between RBC from varicose vein and antecubital vein of the patients. The same was observed for LSF.

DISCUSSION

The influence of PTX and one of its most active metabolites LSF on the deformability and aggregation of red blood cells were investigated *in vitro*. RBC from healthy volunteers and patients with chronic venous disease were used in the study. An impairment of RBC rheological properties in CVD was previously reported (Boisseau & de La Giclais, 2004; Chwała *et al.*, 2009). We decided to undertake an *in vitro* study because *in vivo* there is always a mixture of the parent compound and its metabolites after administration of PTX, which excludes the possibility of examining and comparing the compounds' activity separately. Although PTX is metabolized in RBC (Ings *et al.*, 1982; Nicklasson *et al.*, 2002), the extent of this phenomenon is rather negligible due to the short duration of the *in vitro* experiment.

The present study showed that incubation of RBC with PTX or LSF led to a marked increase of their deformability. PTX significantly improved the deformability of erythrocytes from CVD patients for three values of shear stress, 2.19, 4.24 and 8.23 Pa, whereas for the RBC from healthy controls also for shear stress of 1.13 Pa. Thus, PTX had better rheological effects on erythrocytes from healthy controls than in those from CVD subjects. At the same time LSF enhanced the deformability for all RBC tested for shear stress values of 4.24 and 8.23 Pa. Chwała *et al.* (2009) demonstrated previously that deformability of RBC was higher in subjects suffering from venous disease than in healthy controls. In the present study the beneficial effects of PTX on RBC deformability were greater in erythrocytes whose deformability was lower. It was shown previously that the improvement of RBC deformability by PTX can be attributed primarily to the increase of adenosine triphosphate (ATP) content in RBC (Stefanovich, 1975). ATP is required to maintain the unique biomechanical properties of the RBC membrane (Nakao *et al.*, 1960).

The obtained results are in good agreement with data obtained by previous investigators who examined the effect of PTX on RBC with impaired deformability. Leonhardt and Grigoleit (1977) showed that PTX addition improved RBC deformability under hyperosmolar conditions. Comparable results were obtained by Ehrly (1979). Seiffge and Kiesewetter (1981) investigated the effect of PTX on single red cell deformability. Those authors showed that PTX addition to a Ca^{2+}-treated red cell suspension reduced the medium passage time through a singlepore membrane under a driving pressure gradient. Additionally, Singh and Kumaravel (1996) confirmed that PTX increased the deformability of normal erythrocytes under *in vitro* conditions.

On the other hand, our results are in contrast to those obtained by Cummings and Ballas (1990), who examined the *in vitro* effect of PTX and its major hydroxyhexyl metabolite on RBC deformability. Erythrocytes were obtained from healthy volunteers and patients with sickle cell disease and incubated with different concentrations of drugs for varying time periods. Those authors demonstrated no effect on the erythrocyte deformability of either compound at any concentration or incubation time period. The discrepancy between our results and those of Cummings and Ballas (1990) may be attributed primarily to the nature and origin of the diseased RBC used in the two experiments (sickle cell disease *vs* chronic venous disease) but also to methodology (e.g., temperature of incubation, number of participants). Additionally, the applied measurement techniques and analyzed parameters are not fully comparable. Moreover, the earlier authors could not distinguish between the enantiomers of metabolite 1 (M1), which differ significantly in their potencies.

In the present study a beneficial influence of the compounds tested on the RBC deformability was noted only at low and medium shear stress (mainly 2.19–8.23 Pa). At the other values of shear stress the EI value did not differ significantly between control RBC and those treated with a drug. The shear rate is determined by the diameter of the vessel and the highest shear rates are observed in the smallest vessels. In large vessels RBC elongate in response to shear forces, while in the microcirculation erythrocytes squeeze through capillaries. Hence, the RBC deformability is measured at a range of shear rates. In the elongation index *vs* shear stress curve the initial part of the curve represents the rigidity of the cell membrane (Hardemann & Ince, 1999). In the present study this part of the curve was significantly affected, thus the observed alternations in RBC deformability may be caused by changes in the RBC membrane.

The present study showed that incubation of RBC with PTX resulted in a marked decrease of their aggregation (Fig. 3A–C). These findings are consistent with earlier results obtained by Muravyov *et al.* (2007), who examined *in vitro* hemorheological efficiency of drugs targeting intracellular phosphodiesterase activity. It was found that PTX significantly decreased normal red cell aggregation. In the present study the decrease was comparable for erythrocytes from the antecubital vein of healthy donors and varicose vein of the patients and was about 22,5% at the higher concentration of PTX tested. Additionally, PTX reduced the tendency of RBC from all sources used in the experiments to form aggregates and their stability (Fig. 3D–F). It can be seen, however, that the beneficial effect of PTX on RBC aggregation was most apparent for those cells whose aggregation tendency and aggregate stability were the greatest (RBC from the varicose vein of CVD patients). Furthermore, PTX significantly decreased the rate of aggregation (T½ analysis) of RBC from the varicose veins. This confirms earlier data suggesting that RBC with an impaired aggregation and deformability (due to a disease) may be useful for the *in vitro* evaluation of the rheological activity of

xenobiotics (Dintenfass, 1983; Salbaş *et al.*, 1993; Lipovac *et al.*, 2000).

The mechanism responsible for the beneficial influence of PTX on RBC aggregation may be related to the improvement of RBC deformability (Seiffge, 1982). Moreover, PTX has been reported to reduce fibrinogen level (Angelkort & Kiesewetter, 1981; Perego *et al.*, 1983). The presence of large plasma proteins such as fibrinogen is the major cause of aggregation (Skalak *et al.*, 1981; Marton *et al.*, 2001).

Concerning earlier studies on the *in vivo* effect of PTX on RBC rheological properties, Schneider (1989) examined the hemorheological effects of PTX treatment in patients with cerebrovascular disease. The study showed that in patients who received PTX parenterally erythrocyte aggregation did not change significantly whereas there was a marked improvement in RBC deformability and yield shear stress. Additionally, during oral PTX treatment the hemorheologic variables, which were pathologically altered at baseline, improved significantly. Dawson *et al.* (2002) evaluated hemorheologic effects of PTX and cilostazol administered to adults with moderate to severe claudication on the viscosity, fibrinogen level and RBC deformability. Those authors concluded that *ex vivo* rheologic characteristics of blood from patients with intermittent claudication were not significantly affected by long-term administration of PTX or cilostazol. Additionally, PTX did not modulate RBC deformability. Studies on the efficacy of pentoxifylline treatment indicate that clinical improvement becomes apparent usually two to four weeks after the first oral dose. It follows that the drug should be administered for at least four weeks and discontinued if there is no clinical improvement. This time period appears to be necessary for recompensation of the patho-hemorheologic abnormality in ischemic tissue (Aviado & Dettelbach, 1984).

Regarding the effects of PTX and its metabolite on RBC deformability and aggregation, PTX is rapidly metabolized to a variety of metabolites, denoted M1–M7. After oral or intravenous administration of PTX to healthy volunteers, the plasma levels of metabolite 1 (M1) (including LSF) as well as metabolite 5 (M5) significantly exceeded the level of the parent drug (Beerman *et al.*, 1985; Nicklasson *et al.*, 2002). The present *in vitro* study showed that LSF significantly improved the deformability of erythrocytes and may thus contribute to the *in vivo* haemorheological effects of pentoxifylline. These results are in good agreement with data obtained by Ambrus *et al.* (1995), who demonstrated that M1 and M5 are similar to PTX in their activity on RBC membrane fluidity. In contrast, we found no significant effect of LSF on the aggregation of RBC *in vitro*. Thus, LSF has no contribution to this particular effect of the parent compound. It is plausible that the effects of PTX on erythrocyte aggregation are due to the activity of the other metabolites.

In summary, it was demonstrated that *in vitro* addition of PTX or LSF effectively increased deformability of RBC from healthy donors and patients with CVD. Thus, LSF may contribute to the *in vivo* hemorheological effects of pentoxifylline. On the other hand, there was no significant effect of LSF on aggregation of RBC *in vitro*. Hence, LSF has no contribution to this particular effect of the parent compound. Additionally, the present study demonstrated the usefulness of RBC with impaired deformability and aggregation for the evaluation of *in vitro* rheological activity of xenobiotics.

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